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SILICA-IMMOBILIZED ENZYMES FOR MULTI-STEP SYNTHESIS IN MICROFLUIDIC DEVICES

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14. ABSTRACT

The microfluidic combinatorial synthesis of 2-aminophenoxazin-3-one (APO) is reported. Individual microfluidic chips containing metallic zinc, silica-immobilized hydroxylaminobenzene mutase and silica-immobilized soybean peroxidase are connected in series to create a chem-enzymatic system for synthesis. Zinc catalyzes the initial reduction of nitrobenzene to hydroxylaminobenzene which undergoes a biocatalytic conversion to 2-aminophenol, followed by enzymatic polymerization to 2-aminophenoxazin-3-one. Silica-immobilization of enzymes allows the rapid stabilization and integration of a biocatalyst within a microfluidic device with minimal preparation. The system proved suitable for synthesis of a complex natural product (APO) from a simple substrate (nitrobenzene) under continuous flow conditions.

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Silica-Immobilized Enzymes for Multi-Step Synthesis in Microfluidic Devices

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ABSTRACT: The combinatorial synthesis of 2-aminophenoxazin-3-one (APO) in a microfluidic device is reported. Individual microfluidic chips containing metallic zinc, silica-immobilized hydroxylaminobenzene mutase and silica-immobilized soybean peroxidase are connected in series to create a chemo-enzymatic system for synthesis. Zinc catalyzes the initial reduction of nitrobenzene to hydroxylaminobenzene which undergoes a biocatalytic conversion to 2-aminophenol, followed by enzymatic polymerization to APO. Silica-immobilization of enzymes allows the rapid stabilization and integration of the biocatalyst within a microfluidic device with minimal preparation. The system proved suitable for synthesis of a complex natural product (APO) from a simple substrate (nitrobenzene) under continuous flow conditions.

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KEYWORDS: microfluidics; immobilized enzyme; aminophenoxazinone; chips; sequential catalysis

Introduction

The use of microfluidics and 'lab-on-a-chip' devices for efficiently miniaturizing biochemical assays and chemical synthesis is now well recognized (Barry and Ivanov, 2004; Figeys and Pinto, 2000; Haswell et al., 2001; Mitchell, 2001; Polson and Hayes, 2001; Urban et al., 2006). The dramatically increased surface to volume ratios lead to rapid mass transfer and greatly reduced analysis time and sample consumption. There is increasing interest in the integration of enzymes into microfluidic systems for applications in medical diagnostics, biosensing and natural product and organic synthesis (Hadd et al., 1997; Jones et al., 2004; Krenkova and Foret, 2004; Ku et al., 2006; Lee et al., 2003; Srinivasan et al., 2003, 2004). Such biocatalytic systems are typically enabled by covalent attachment of the biocatalysts to channel walls, physical absorption onto solid matrices, or copolymerization (Holden et al., 2004, 2005; Honda et al., 2005; Mao et al., 2002; Sakia-Kato et al., 2004). Biocatalysts encapsulated in sol-gels and hydrogels can be subject to leaching, which reduces the active enzyme concentrations (Sakai-Kato et al., 2003). We recently reported a method of enzyme immobilization in biomimetic silica that increases the mechanical stability of the immobilized enzyme and facilitates application to flow-through reaction systems (Berne et al., 2006; Luckarift et al., 2004, 2006). The silica immobilization method is simple, rapid, and applicable to a wide variety of enzymes. The strategy precludes the need to modify the surface to allow enzyme attachment, greatly reducing preparation time and enhancing the loading capacity of the reaction system.

In this study we investigated the applicability of silica-immobilized enzymes for entraining biomolecules in microfluidic devices, and in the process developed a functional chemoenzymatic microfluidic platform. Specifically, with nitrobenzene (NB) (1) as a model substrate we demonstrate a three-step continuous reaction system for the production of 2-aminophenoxazin-3-one (APO) (4) (Fig. 1). In the first step, metallic zinc reduces NB (1) to hydroxylaminobenzene (HAB) (2) (Furniss et al., 1989), which then undergoes an enzymatic intramolecular rearrangement catalyzed by HAB-mutase to form 2-aminophenol (2-AP) (3) (Davis et al., 2000; Luckarift et al., 2005; Nadeau et al., 2003). In the final stage, soybean peroxidase

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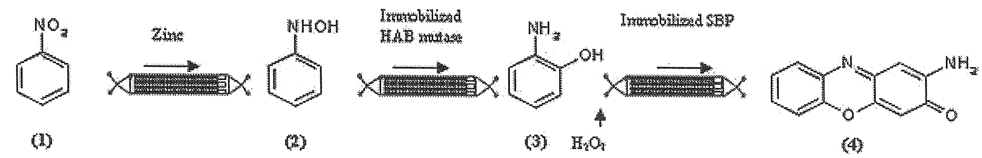

Figure 1. Schematic of a multi-step microfluidic process for the conversion of nitrobenzene to 2-aminophenoxazin-3-one.

(SBP) catalyzes the oxidation of 2-AP to form APO (4) (Fomsgaard et al., 2004; Hishida et al., 1974; Horvath et al., 2004; Lee et al., 2003; Reihmann and Ritter, 2002; Simandi et al., 2004; Srinivasan et al., 2003; Toussaint and Lerch, 1987) APO is an intermediate in the synthesis of actinomycins; an important group of antibiotics with anti-fungal and anti-tumor properties (Barry et al., 1989; Shimizu et al., 2004; Veselkov et al., 2003).

Materials and Methods

Materials

Hydroxylaminobenzene was synthesized as described previously (Furniss et al., 1989). Partially purified HAB mutase A was prepared and immobilized as described previously (Luckarift et al., 2005). All other chemicals were of analytical grade and obtained from Sigma–Aldrich (St. Louis, MO).

Fabrication of Microfluidic Chips

Standard photolithographic and molding techniques were used to fabricate a microfluidic channel (40 mm long × 1.5 mm wide × 0.1 mm deep; volume of 6 μL) in polydimethylsiloxane as described previously (Ku et al., 2006; Srinivasan et al., 2004). The resulting chip is fabricated with three 50 μm channels to form an exit network to retain the immobilized catalysts. Silica-immobilized biocatalysts were loaded into the channel by applying a vacuum to the product reservoir. HAB-mutase enzyme activity was determined as described previously (Davis et al., 2000). SBP enzyme activity was determined with ABTS (2, 2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) and hydrogen peroxide as described previously (Amisha Kamal and Behere, 2003).

Chip-Based Synthesis

All substrates were dissolved in water containing NH_4 Cl (40 mM). Substrates were pumped at fixed flow rates and the eluate collected in an equal volume of acetonitrile for HPLC analysis. Reactants and products of all conversions were monitored by reverse-phase HPLC on a Supelco ABZ column with an acetonitrile/water gradient as described previously (Luckarift et al., 2005).

Synthesis of 2-Aminophenoxazin-3-One Product Standard

2-Aminophenol (100 mg in 1L of potassium phosphate buffer, pH 7.4) was incubated with 0.01 U/mL SBP enzyme and 0.6 mM H_2O_2. A deep red product characteristic of APO formed rapidly and precipitated from solution. The product was purified by solid phase extraction and was characterized and identified by NMR as 2-aminophenoxazin-3-one by comparison to proposed chemical shifts and previous literature reports (Gabriele et al., 2003). The yield of the reaction was approximately 83% and the product was used as a standard for future reactions. NMR spectra were recorded on a Varian Inova spectrometer equipped with a 5 mm indirect detection probe, operating at 500 MHz for ^1H and at 125 MHz for ^{13}C.

Results and Discussion

Biosynthesis of APO in a Microfluidic Chip

Silica-immobilized SBP was prepared and demonstrated good retention of enzyme activity (65–85%) relative to the soluble enzyme. The silica-immobilized SBP was packed into a microfluidic channel with an equal volume of agarose beads (1:1 vol/vol), to give a final enzyme loading of approximately 0.5 U. The presence of agarose beads prevents the silica particles from packing and reduces the void volume of the microfluidic reactor (effective volume ~3 μL). 2-AP (0.5 mM) was pumped through the SBP chip at a range of flow rates and the conversion efficiency of the enzyme was determined by measuring production of APO. Hydrogen peroxide (0.5–2.5 mM in 10% DMF) was added continuously as a second reactant for the oxidation reaction. A molar ratio of 2.5:1 H_2O_2:2-AP was optimal for conversion of 2-AP to APO (Fig. 2). The initial reaction velocity of immobilized SBP was 83 and 98 μM/min at 1.0 and 2.5 mM H_2O_2, respectively.

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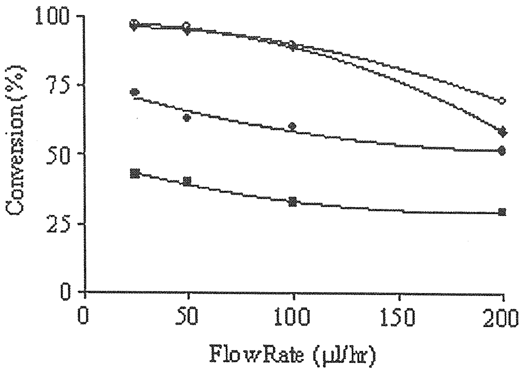

Figure 2. Effect of H_2O_2 concentration on conversion of 2-AP to APO in a silica-immobilized SBP chip. Conversion efficiency of 2-AP catalyzed by SBP with 2.5 mM (○), 1 mM (◆), 0.5 mM (●) and 0.1 mM (■) H_2O_2.

Biosynthesis of 2-Aminophenol in a Microfluidic Chip

Immobilized HAB-mutase demonstrated good retention of enzyme activity relative to soluble enzyme, with little variability among replicate preparations (45–65%). Silica-immobilized HAB-mutase was mixed with an equal volume of agarose beads and packed into the channel as described above to give a final enzyme loading of approximately 1 unit (U). HAB (1 mM) was pumped through the HAB-mutase chip at a range of flow rates and the conversion efficiency determined. Conversion efficiency decreased with increasing flow rate as expected due to the decreased residence time (Fig. 3). The maximum 2-AP product concentration obtained from 1 mM HAB was 0.61 mM (±0.09) at a flow rate of 50 µL/h. The reusability of the silica-immobilized HAB-mutase within the microfluidic chip was also determined to verify the retention of the biocatalyst within the channel during continuous flow. The enzyme chips were washed and stored repeatedly at 4°C and retained >50% of the original enzyme activity over a number of subsequent cycles (Fig. 4).

Biosynthesis of APO From HAB

The two immobilized-enzyme chips (HAB-mutase and SBP) were connected in series and an aqueous solution of HAB (1 mM) was introduced at a flow rate of 100 µL/h. This resulted in the production of APO (0.13 mM ±0.025) continuously for over 4 h (conversion efficiency of ~52%) (Table I). The addition of H_2O_2 to the system diluted the substrate concentration and reduced the theoretical product concentration by 50%. The flow rate could be increased to 150 µL/h, however, with no loss in conversion efficiency.

Chemoenzymatic Synthesis of APO

To complete the chemoenzymatic microfluidic platform, a metallic zinc chip was prepared (1 mg zinc dust, mixed 1:1 with agarose beads) and attached prior to the two enzyme reactors to demonstrate a combinatorial chemoenzymatic conversion of nitrobenzene to the resultant phenoxazinone. The zinc chip gave approximately 25% conversion of NB to HAB at low flow rates. In contrast to the chips containing biocatalysts, the metal zinc chip showed increased conversion efficiency with increasing flow rate (Fig. 3). At low flow rates, aniline was formed as a byproduct of the zinc reduction in preference to HAB. As a consequence, when the zinc chip was added in series with the two enzyme chips and NB pumped at a flow rate of 150 µL/h, the overall conversion efficiency was reduced to 19% due to the reduction in the corresponding efficiency of the zinc conversion step (Table I). Reduced conversion efficiency was also attributed to absorption and volatility

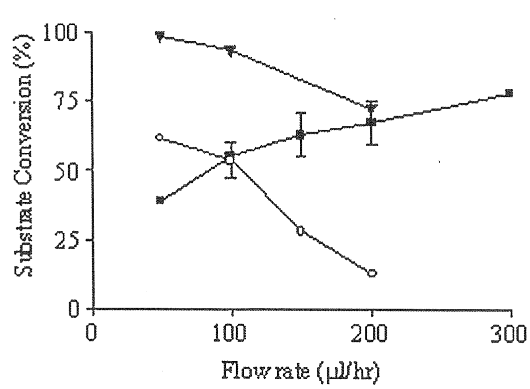

Figure 3. Substrate conversion efficiency as a function of flow rate. Conversion efficiency of 1 mM NB by Zn (■), 1 mM HAB by HAB-mutase (○) and 1 mM 2-AP by SBP and 1 mM H_2O_2 (▼).

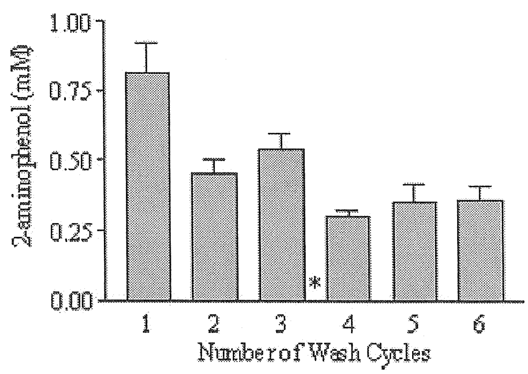

Figure 4. Reusability of HAB-mutase chips over several wash cycles. HAB (100 mL, 1 mM) was pumped through the HAB-mutase chips at a flow rate of 150 µL/h and the formation of 2-aminophenol was determined. The chips were washed with 20 volumes of buffer between each subsequent cycle. Data shown is the mean and standard deviation of three independent HAB-mutase chips. Chips were stored overnight at 4°C at the point indicated (*).

Table I. Conversion efficiency of multi-step microfluidic synthesis.

Substrate	Chip combination	Conversion efficiency (%) 100 μL/h	150 μL/h
1 mM NB	Zinc/HAB mutase	16.7 ± 3.5	28.2 ± 2.7
1 mM HAB	HAB mutase/SBP	53.2 ± 8.8	51.7 ± 10.2
1 mM NB	Zinc/HAB mutase/SBP	ND	18.9 ± 10.14

ND, not determined.

of the substrate. In an empty chip, observed NB concentrations were reduced in the eluate relative to the starting concentration but the percentage overall loss decreased with increasing flow rate. The conversion of the chemoenzymatic system was, therefore, determined at flow rates considered optimal for the biocatalytic steps. Clearly the step involving conversion of the nitro compound to the corresponding hydroxylamino compound will require optimization for future applications. Despite the low overall yields of the chemoenzymatic steps in series, the system provides a rapid and versatile method for screening the conversion of nitroarenes.

In this work, we demonstrated the first chemoenzymatic microfluidic reactor for the synthesis of a natural product from a readily available and simple substrate, nitrobenzene. The facile silica-immobilization method provides stable heterogeneous catalysts, which can be easily incorporated into microfluidic chips. The flow-through system described could be applied to the transformation of a wide variety of nitroarene substrates into their corresponding phenoxazinone products, and provides an attractive alternative to conventional chemical synthesis. The versatility of the immobilization method makes the system amenable to a wide range of microfluidic devices incorporating biomacromolecules and coupled to chemical synthesis. Single unit reactors containing enzyme or metal catalysts can be integrated to create a variety of synthetic pathways for rapid synthesis and screening.

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